Extracellular vesicles swarm the cancer microenvironment: From tumor-stroma communication to drug intervention

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Abstract

Intercellular communication sets the pace for transformed cells to survive and to thrive. Extracellular Vesicles (EVs), such as exosomes, microvesicles and large oncosomes, are involved in this process shuttling reciprocal signals and other molecules between transformed and stromal cells including fibroblasts, endothelial and immune cells. As a result, these cells are adapted or recruited to a constantly evolving cancer microenvironment. Moreover, EVs take part in the response to anticancer therapeutics not least by promoting drug resistance throughout the targeted tumor. Finally, circulating EVs can also transport important molecules to remote destinations in order to prime metastatic niches in an otherwise healthy tissue. Although the understanding of EV biology remains a major challenge in the field, their characteristics create new opportunities for advances in cancer diagnostics and therapeutics.

EVs at the interface of stromal communication

Instigated by malignant cells the surrounding stroma undergoes a shake-up in its organization that supports cancerous growth. Crucial parts of this self-organization process include induction of metabolic changes, modifications of cell identities, initiation of neo-vascularization and reprogramming of inert immune cells. In order to achieve these defining properties of the tumor microenvironment, cancer and non-cancer cells continuously exchange information brought together through cell-cell traversing gap junctions, tunneling nanotubes, and the secretion of effector molecules. One way to guarantee coordinated release of multiple "game" changing molecules relies on their packaging into membrane enclosed vesicles widely known as extracellular vesicles (EVs). "EVs" is a general term coined to denominate vesicle carriers that in fact hugely differ in their subcellular origin (**Figure 1**). They contain cargo such as lipids, proteins, various RNAs and DNA fragments and metabolic products. EVs may shuttle these molecules between neighbouring cells or via systemic transport to distant anatomic sites where they may induce signaling pathways or directly alter the phenotype of specified recipient cells.

One kind of EVs finds its origin in secretory multi-vesicular bodies that fuse with the plasma membrane releasing intraluminal vesicles, thereafter called exosomes (50-150nm in diameter). Another kind of EVs derive from vesicle budding at the plasma membrane. These are commonly called microvesicles (MVs) and are more heterogeneous in size (>100nm-1μm in diameter). Finally, large oncosomes (LOs, >1μm) have been described that differ in their buoyant density from the aforementioned vesicle types, are produced by plasma membrane blebbing (reviewed in [1, 18]). All of those nanovesicles can be found in and isolated from

conditioned tissue culture medium of cancer and stromal cells but also from diverse body fluids such as cerebrospinal liquid, breast milk, urine or blood plasma.

Due to their cargo specificity and their easy sourcing circulating EVs are being evaluated for the early diagnosis of various cancers. Indeed, EV cargo such as survivin may serve as marker for the early diagnosis of prostate cancer[37], caveolin-1 for melanoma[37], Glypican-1 for early pancreatic cancers[50], and various miRNA profiles in colorectal cancer[57] and lung cancer[12].

EVs have recently also been implicated as direct mediators of the response of solid tumors to cytotoxic chemotherapy, and as putative 'real-time' biomarkers to assess individual drug responses. The evidence demonstrating modulation of drug sensitivity has centered on the EV-mediated transfer of proteins, mRNAs and miRNAs with the capacity to influence key anti-apoptotic or proliferative pathways between tumor cells or from the endothelium to tumor cells (see below).

Navigating across these different aspects, this review will focus on the latest functional insights that EVs bear in intercellular communication during cancer progression.

Modulation of EV composition

Both exogenous as well as endogenous factors can modulate type, content and the number of released EVs. As discussed in more detail further below, hypoxia appears to be a strong driving force in the enhancement of EV shedding, resulting in pro-angiogenic effects. Furthermore, intra-tumoral hypoxic conditions augment MV release leading to increased risks of metastasis and mortality in patients with advanced breast cancer[80]. PH changes in the tumor microenvironment can also contribute to changes of the lipid composition of EVs[62]. In

addition, the cellular stress regulated protein TSAP6 that is under the control of the p53 tumor suppressor was shown to enhance exosome production with possible effects on adjacent cells and the immune system[82]. Although our understanding of changes observed in EV composition under different physiological conditions is still minimal, they nevertheless may pave the way to novel, exciting avenues in diagnosis and treatment of cancers.

In breast cancer for instance, the overexpression of oncogenes such as ERBB2/HER2 in the mammary luminal epithelial cell line (HB4a) can shift the bias of EV content towards a malignant phenotype, as defined by the detection of oncodriver signaling components, including HER2, cell adhesion and cytoskeleton-remodeling components and sphingosine-1-phosphate[5]. Similarly, oncogenic Ras-transformed NIH3T3 cells showed an increase of over 34 proteins in EVs, including milk fat globule EGF factor 8 (lactadherin), collagen alpha-1 (VI), 14-3-3 isoforms, guanine nucleotide-binding proteins (G proteins), the eukaryotic translation initiation factors elF-3 gamma and elF-5A accumulated (>2-fold)[34]. Mutated KRAS in colon cancer cells has also been reported to effect EV cargo composition towards tumor promoting factors including mutated KRAS itself as well as EGFR, SRC family kinases, and integrins, when compared to its isogenically matched wild-type KRAS cells[17]. Importantly, mutant cell-line-derived EVs positively enhanced cell growth of wild type cells[17]. Another oncogene, the melanoma cells secreted Wnt5A was also reported to induce the release of EVs[20]. Finally, tumorigenic viruses such as EBV can manipulate the secretion of EV bound cellular components, namely integrins, actin, IFN, and NFκB that subsequently activate cellular signaling in the surrounding stroma[49].

Although we now have evidence that oncogenes can directly modify cargo load, the knowledge of its consequences is still stuck in its infancy.

EVs reprogram cancer cell metabolism

The development of cancers as a multi-stage process is often ignored in *in vitro* studies. As a result, we obtain a picture of cancer signaling and oncodriver activity that is blind to the spatiotemporal context of our observations, leaving us with the egg-chicken problem. EV composition presumably reflects the cellular physiology of their parent cells and can transport 'seeding' information to recipient cells. This implies that EVs carry the capacity to reprogram the cellular metabolism and re-wire cellular interactions (**Figure 2**). Therefore, EVs provide the rare opportunity to analyze the direct and causal effect that fractionated information has on oncogenic transformation.

In this context, it is useful to understand that during the lifetime of a solid tumor its cells are subjected to enormous microenvironmental shifts, some of which are large enough to induce permanent transformations, may these be post-transcriptional and/or epigenetic or indeed metabolic, such as the Warburg effect. Additionally, during cancer development cell populations become increasingly heterogeneous. The extent to which an initial population is clonally diverse is still under debate; however, a hostile environment prompts malignant cells to adapt, primarily, by changes to their metabolic profiles, thus reprogramming the energetics of biosynthesis. For instance, the effect of hypoxia on HIF-1α, carbonic anhydrases (such as CAIX), the sodium/proton exchanger NHE1 and the glucose transporter Glut1 have been portrayed exquisitely in most solid tumors and paved the way for the discovery of metabolite import/export pumps demonstrating cancer cell plasticity by recycling their "waste material". The best understood of such systems is provided by the proton-lactate symporters belonging to the family of monocarboxylate transporters (MCTs)[27, 28] and their co-chaperone, the

glycoprotein CD147[42]. Under regimes of high glycolytic flux, lactic acid is initially exported in response to intracellular pH regulators. These alter cellular acid export providing the cell with an alkaline pH that in turn favors glycolysis and the import of glucose. However, the acidic burden resulting from glycolysis can eventually result in toxicity prompting the emergence of invasive cells[81]. Lactate can then be re-imported through the MCTs, a process known as lactate shuttling, and used as a source of energy in OXPHOS active cells via the lactate dehydrogenases (LDHA/LDHB) that convert lactate to pyruvate[64]. It is of great interest, therefore, that exosomes have been shown to contain high levels of Glut1, MCT4 and CD147 as well as reduced phosphoglycerate kinase (PGK) levels[68] because this finding appears consistent with the key elements characterizing the "reverse Warburg effect" shown to occur in stromal cells. In this scenario, metabolic EV content could 'highjack' the existing cellular program and re-wire it, presumably mimicking the cell of origin. The uptake and release of EVs is considered an energetically unfavorable event; cancer cells notoriously show reduced or lack of OXPHOS-derived ATP, elicit increased reliance on glycolysis, the pentose phosphate pathway and alternative energy sources such as lactate and acetate. However recent evidence has shown that EVs originating from prostate cancer cells can actually produce ATP from glycolysis and show reduced ATPase activity, when compared to EV populations released by normal prostate tissue (or prostasomes)[68], making their reception, rather than their release, the energetically favorable event. In many ways, EV formation by cancer cells appears more similar to an energetic investment made towards future re-homing by outsourcing their energy requirements. It would be of significant interest, and presumably possible, to re-engineer this machinery in the opposite direction and deliver tumor suppressor information from the microenvironment (such as fibroblasts, T-lymphocytes or neutrophils) to the cancer cells. Instead, cancer associated fibroblasts (CAFs)

-derived EVs shuttle a range of metabolites to prostate and pancreatic cells, including lactate, glutamine, lipids, TCA cycle intermediates, resulting in reduced OXPHOS and increased reliance on glutamine and glycolysis[84]. This is at odds with the current understanding of metabolic reprogramming being an autonomous event occurring in cancer cells in response to nutrient deprivation. In this light, it appears that metabolic re-wiring is enhanced and could even be initiated by the tumor microenvironment, questioning much of the theoretical framework elaborated to explain malignant transformation and progression.

KRAS activating mutations have been associated with oncodriver activity along the MAPK signaling pathway and have recently been shown to drive a glycolytic switch in NSCLC cells[36]. During PanIN de-differentiation KRAS mutations in acinar cells have been shown to drive PKD1-dependent mitochondrial ROS increases and that this event is the leading factor responsible for EGFR-mediated ADAM17 shedding[43]. Similarly, in KRAS mutant colorectal cancer, inhibition of the PI3K/mTOR pathway sensitizes cells to EGFR inhibitors[8]. Indeed, it has been reported that some EV populations form through DAG-controlled fission and the secretion of which is dependent on the combined action of DGKα, which releases phosphatidic acid from diacylglycerol (DAG), and PKD1/2[48]. Furthermore, pancreatic cancer patient-derived EVs contain oncogenic KRAS and subsequent analysis showed that the KRAS mutation status of EVs matched the primary tumor[50]. It is reasonable to hypothesize that EVs shuffle a diverse pool of signaling elements belonging to the KRAS pathway, as well as metabolites such as DAG, lactate and glutamine satisfying sufficient requirements to drive malignant transformation in healthy recipient cells. Proteomic profiling of EVs using stable isotope labeled amino acids in cell culture (SILAC) has further shown that exosomal cargo content is dependent on vesicle size[52]. LOs preferentially contain protein cargo targeted to mitochondrial metabolic processes including VDAC1/2, the solute carriers

SLC25A6 and SLC25A5 that are mitochondrial ADP/ATP translocators as well as the ATP synthase subunit ATP5B. Nano-sized EV cargo on the other hand contained higher amounts of proteins clustered towards glucose and glutamine metabolism and gluconeogenesis[52]. Because EV content seems size-dependent it is plausible that release and uptake of small EVs are coordinated separately from LOs. Cholesterol flux and functional lipid rafts affect the uptake of EVs in A375 melanoma cells[63]. We speculate that these and other mechanisms may in part help explain why certain cargo is tailored in an organotropic manner, thus favoring a tissue-specific metastatic phenotype. Metabolic reprogramming under stress appears to be one of the primary functions of EVs and HIF-1α has been detected in nasopharyngeal carcinoma exosomes where LMP1-induced transmission of transcriptionally active HIF-1α drives oncogenic processes[2].

Our current understanding of metabolic reprogramming events during cancer development is still widely elusive; in particular, the spatiotemporal order with which cells undergo metabolic reprogramming has not been fully evaluated. Further characterization of the feedback loops initiated by EVs on tumor cells and the stromal environment might provide critical missing pieces in this picture.

Stromal effects of EVs

EVs mediate fibroblasts and cancer cell changes

Fibroblasts make up the bulk of stromal cells. Although hugely variable, even within the same kind of tumor, fibroblasts are in most cases the main contributor to the stroma. For instance, in invasive ductal carcinoma the average number of fibroblasts/myofibroblasts may reach up to 50-70% of the total stroma cell number. TGFβ, PDGF and FGF2 signaling-

ligands in conjunction with other molecules including miRNAs can induce a cancer-activated or associated fibroblasts (CAFs)/myofibroblast phenotype characterized by increased proliferation rate, migratory properties and heightened deposition of ECM. CAFs originate from resident fibroblasts, through induction of epithelial-to-mesenchymal transition (EMT) or via recruited and reprogrammed mesenchymal stem cells (MSCs) and produce several growth factors such as HGF, VEGF and TGF[35]. Breast cancer cells (BCCs)-derived TGFβ-EVs show the ability to differentiate adipose tissue-derived MSCs into α-smooth muscle actin positive CAFs utilizing the TGFβ/Smad pathway[15]. Furthermore, prostate cancer-derived EVs may induce CAFs from bone-marrow MSCs with pro-angiogenic and invasive functions[16]. This could be in part explained by the abundance of miR-1227 in LOs from the prostate cancer cell line RWPE-2 that enhances CAF migration properties[55]. EVs appear to induce CAFs, as recently substantiated by the findings that bladder cancer-derived EVs induce EMT in urothelial cells[23]. However, EVs from non-solid cancer chronic lymphocytic leukemia can also turn stromal endothelial cells and MSCs into CAFs[60]. On the other hand, stromal cells themselves are known to secrete EVs. In a human/mouse tissue culture system, Wnt11-EVs activated the Wnt-planar cell-polarity signaling pathway at the leading edge of BCCs eliciting cell migration. In that case, cancer cells and fibroblasts work together to assemble fibroblast EVs that are internalized by BCCs, loaded with Wnt11 protein and then re-released for paracrine signaling[45].

In a different context CAF EVs with increased levels of miRNA-21 profoundly impact ovarian cancer growth by suppressing apoptosis through binding to its novel target, APAF1[7]. Finally, as discussed above CAF-derived EVs directly participate in metabolic reprogramming. In aggregate, these few examples add to an increasing number of described EV functions in bidirectional cell interactions between fibroblasts and cancer cells.

EVs set the place and time for neo-angiogenesis

Neo-angiogenesis allows tumors to get their own constant vascular supply of nutrients and oxygen, enabling them to grow above $2mm^3$ and become much more aggressive. One of the most recent advances in this field is the involvement of EVs in tumor-associated neo-angiogenesis[24, 61]. Indeed, several groups reported the pro-angiogenic effect of tumor cell-derived EVs on endothelial cells in different types of cancer such as glioblastoma[71], leukaemia[74], melanoma[31] and ovarian cancer[51]. Since EVs can be taken up by endocytic-like processes, they may evade the ligand-receptor system on the cell surface influencing intracellular signaling and protein expression in endothelial cells[25]. As mentioned above, EVs can exert functions over short and long distances. In this way, pro-angiogenic EVs influence the neo-angiogenic program in the proximal tumor microenvironment but can also prime metastatic niches for angiogenetic events[26, 31].

Pro-angiogenic factors such as VEGF, FGF, PDGF, interleukins, matrix metalloproteinase (MMPs), EGFR or signaling proteins including Rac1, Cdc42/Pak2 can be found among other proteins in tumor cells-derived EVs[25, 41, 71, 78]. The presence of these proteins in EVs brought novel aspects of tumor-associated neo-angiogenesis into the limelight. For instance, Al Nedawi et al. reported that upon uptake of tumor cell-derived EVs that contained oncogenic EGFR, endothelial cells establish a VEGF-dependent autocrine loop, a main mechanism in tumor neo-angiogenesis[3]. Such a process re-programs endothelial cells and consequently, strongly enhances neo-angiogenesis. More recently, Gopal et al showed that tumor cell-derived EVs are able to deliver signaling factors, such as Rac1 or

Pak2, or receptor proteins such as neuropilin 1, a co-receptor for VEGF, directly to endothelial cells promoting neo-angiogenesis[25]. In comparison to the classical "ligand/receptor" process, the authors called this phenomenon a "more direct avenue to induce angiogenesis" and suggest that it could be involved in metastatic spread[25] (**Figure 3**).

Some mRNAs and miRNAs found in EVs are thought to bespecifically involved in neo-angiogenesis[78]. For example in colorectal cancer, tumour-derived EVs can promote proliferation of endothelial cells and enhance their cell-cycle activities through M-phase related mRNAs, such as those coding for the centromere protein E (CENPE), PDZ binding kinase (PBK) or cyclin-dependent kinase 8 (CDK8)[32]. Additionally, the involvement of vesicular miRNAs in neo-angiogenesis has been studied such as miRNA-210 that exhibited strong pro-angiogenic activity[22, 40, 83]. Furthermore, miRNA-210 has been observed to suppress the expression of specific genes such as EFNA3 (coding for Ephrin-A3) in endothelial cells, resulting in enhanced neo-angiogenesis[21, 39, 73]. Colorectal carcinoma cells-derived vesicular miRNA-9 shows pro-angiogenic effects through inhibiting the expression of suppressor of cytokine signaling 5 (SOCS 5), promoting the activation of the janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling, a driver of endothelial cell migration[85]. Leukemia cells-derived exosomal miRNA-92a has also been shown to stimulate tumor associated neo-angiogenesis, through the inhibition of integrin α5 expression[77].

Despite the direct pro-angiogenic effect of cancer cell-derived EVs on endothelial cells, such vesicles also promote neo-angiogenesis through indirect effects on other stromal resident cells. For example, leukemia-derived EVs can induce a CAF phenotype in stromal cells in the surrounding microenvironment, hence leading to increased expression of pro-

angiogenic factors in such cells[40, 60]. Finally, EV-mediated crosstalk occurs also between endothelial cells themselves[79].

On the other hand EVs may act on tumor cells during neo-angiogenic processes since endothelial cells themselves have been shown to release EVs that can target tumor cells. Indeed, endothelial HUVEC cells were shown to secrete EVs containing miRNA such as miRNA-503 that were taken up by co-cultured tumor cells *in vitro*. MiRNA-503 was subsequently linked to response to neo-adjuvant chemotherapy in breast cancer[9].

Several reports suggested that the increased number of tumor cells-derived EVs during neo-angiogenesis could be a reaction to a hypoxic condition, a key event in promoting neo-angiogenesis[21, 62, 72]. In addition, recent data showed that the composition of EVs may also depend on the hypoxic status of glioma cells[41]. In using glioma cell lines and patient-derived cells EV signature composition was positively correlated to hypoxia. This led to the observation that hypoxic tumor cell-derived EVs are more potent neo-angiogenesis inducers than EVs-derived from normoxic populations. Interestingly, hypoxic tumor cell-derived EVs execute this function by PI3K/Akt signaling modulation[41]. Furthermore, vesicular miRNA-135b from hypoxic multiple myeloma cells can directly contribute to enhanced neo-angiogenesis under chronic hypoxia through the inhibition of the factor inhibiting hypoxia-inducible factor 1 (FIH-1) expression, promoting the activity of HIF-1[77]. Other groups also reported on special selection processes for proteins and RNA content of tumor cell-derived EVs in response to hypoxia, providing them with specific pro-angiogenic functions[38, 67, 69, 73]. Finally, WNT5A signaling protein induces mechanisms that lead to the release of EVs from tumor cells containing pro-angiogenic factors such as VEGF[20].

These data also suggest that different tumor types can release different EVs with variable outcome for neo-angiogenesis. For instance, tumor cells undergoing complete epithelial-mesenchymal transition (EMT) release EVs that are more effective at enhancing neo-angiogenesis than those undergoing intermediate EMT[25]. Similarly, for renal cancer, EVs with the most powerful pro-angiogenic activity were those derived from cancer stem cells (CSCs) and contained different angiogenic factors, compared to non-CSCs[51].

EVs tune the immune response.

EVs, as mediators of intercellular communication, can modulate the activity and therefore the nature and vigor of diverse cellular immune response systems. Early data demonstrated the ability of Dendritic cell (DC)-derived EVs to stimulate an anti-tumor immune response as well as documented the presence of key MHC1 and MHCII proteins in EVs[86]. More rigid functional evidence of intercellular shuttling of miRNAs with the ability to epigenetically effect target genes in a variety of DC cells was first obtained from EVs from different DC populations that showed varying miRNA signatures depending on their maturation state[54]; miRNA transfer has been demonstrated in both *in vitro* and *in vivo* settings and can effect a range of diverse processes. Transmission occurs sometimes in a unidirectional fashion for instance at the immune synapse from T-cell to antigen presenting cell, in an antigen driven fashion[53]. T-cell derived exosomes containing specific miRNA signatures have been recently shown to suppress T-H1 mediated immune responses in systemic diseases[59]. There is now a growing body of evidence that suggests that cancer cells use EV transmitted nucleic acids and proteins as a way of enacting an immune escape.

Colorectal cancer cell-derived MV content such as TRAIL and FAS ligand has been demonstrated to induce T-cell death through the activation of the FAS-ligand[32]. This has also been demonstrated for other tumor types[6]. In the context of hepatocellular carcinoma the release of heat-shock protein chaperones from EVs was shown to act as a decoy enabling a NK cell response to be directed away from tumor cells. In contrast, in resistant cell lines these HSP bearing EVs were upregulated[46]. Circulating EVs in breast cancer similarly enable tumor growth by downregulating NK cell activity[44]. Tumor-derived EVs in nasopharyngeal cancer were found to induce T-reg activity and inhibit T cell proliferation in vitro.

Whilst the above examples demonstrate that tumor-derived EVs can downregulate the immune response it appears that EVs from activated immune cells can also influence the tumor phenotype. For example, EVs from activated CD8+ T-cells can increase tumor immunogenicity by activating ERK and NFκB signaling through TNF-related signaling leading ultimately to the upregulation of MMP-9. This chain of events increases the metastatic potential in melanoma and lung cancer[11]. In another chain of events pancreatic ductal adenocarcinomas cell-derived EVs can lead to pre-metastatic niche formation in sequential steps of induction of TGFβ signaling in Kupffer cells leading to extracellular matrix modification and subsequently an influx of bone marrow-derived macrophages to the liver, providing a favorable niche for liver metastasis[19].

EVs as 'real-time' biomarkers during cancer therapies

Some of the most promising studies involving EV cargo modulation during drug treatment have been performed in glioblastoma multiforme (GBM). Levels of the DNA repair

enzymes APNG and MGMT are inversely correlated to response to the gold standard chemotherapeutic temozolomide[30]. EVs containing MGMT mRNA have been demonstrated to accurately reflect the levels of these enzymes in parental cells and in patients throughout treatment and therefore could serve as a potential 'real-time' biomarker of chemotherapy response during drug treatment[70]. Similarly, circulating EVs containing the EGFRvIII splice variant that is thought to be predictive of response to EGFR inhibition were detectable in the serum of GBM patients but not in the 30 matched controls[71].

In the context of the neo-adjuvant treatment of breast carcinoma, elevated levels of the EV-bound MDR-glycoprotein BCRP were detected in non-responders compared to responders or treatment naïve patients[14]. In addition, the receptor channel protein TRCP5, a known regulator of multidrug resistance glycoprotein-P, was required for EV formation in anthracycline resistant breast carcinoma cell lines. Moreover, EVs containing TRCP5 protein from the same chemoresistant cells can enter chemosensitive cells and transmit resistance to cytotoxic chemotherapy. The same group also demonstrated elevated levels of TRCP5 mRNA in circulating EVs from patients who did not respond to chemotherapy[47].

Horizontal transfer of nucleic acids has been postulated as one mechanism that can alter apoptotic and proliferative cell responses during cancer treatment. Indeed, EVs from triple negative breast cancer cells *in vitro* can evoke proliferative and angiogenic properties in recipient cells that are similar to those seen in the parental cell line[56]. A recent study elaborating on this work additionally demonstrated transfer of miRNAs including mir-100, miR-222, miR -17 and miR-30a through exosomes in breast cancer cell lines with the effect of modulating target genes which can be critical to drug response. For instance the transfer of miR-222 specifically caused PTEN mRNA downregulation in recipient cells. The subsequent apoptotic response to doxorubicin was also reduced[13]. In addition to miRNAs,

proteins transported by EVs have also been shown to modify the apoptotic response. The key negative regulator of AKT/PI3 kinase signaling PTEN for instance has been identified as EV cargo eliciting active phosphatase function in the recipient cell[65].

Only a few studies have been published on the role of EVs in modulating a response to more specific targeted treatments. One such study explored the role of EV transfer between cetuximab resistant and sensitive colorectal cancer cell lines *in vitro*. Although an effect on cell viability was observed, this effect turned out to be rather modest[66]. Recently, IncARSR (Inc RNA Activated in renal cell carcinoma (RCC) with Sunitinib Resistance) has been shown to promote sunitinib resistance via its EV bound transfer to sensitive RCC cells where it competitively binds miR-34/miR-449. Decreasing the levels of those miRNAs facilitates AXL and c-MET expression in RCC cells, rendering IncARSR as a hopeful predictor for sunitinib resistance. Although these few examples seem quite promising it remains widely unexplored and elusive whether EVs are indeed significant contributors to either intrinsic or acquired resistance to the plethora of FDA-approved small molecule inhibitors currently in clinical use.

For anti-angiogenic therapies more data are available overall concluding on positive effects of EVs in modulating drug response. Raimondo et al. analyzed the occurring changes in EV composition and evaluated their effects on drug treatment responses[67]. Interestingly, angiogenic factors present in EVs correlated with patients that were likely to benefit from a particular anti-angiogenic therapy. In addition, EV-dependent mechanisms could be implicated in the refractoriness of some tumor cells to current anti-angiogenic therapies, as observed for glioblastomas in response to bevacizumab. Finally, anti-angiogenic therapies could alter the pro-angiogenic properties of EVs, suggesting this as a new strategy to

decrease tumor-associated vasculature and tumor resistance[29]. Taken together the interference with EV communication could potentially have a strong anti-angiogenic effect[3, 40].

Studying EV-based therapies, some groups have explored the utilization of EVs as therapeutic delivery systems. Taking advantage of EVs in delivering specific RNAs designed to alter the phenotype of malignant cells could prove an attractive prospect. Such a prospect was successfully executed by engineering let-7a miRNA containing EVs to modify EGFR expression in breast cancer cell lines leading to dramatic effects on tumor growth[58]. Similarly, delivery of extrinsically administered siRNA using exosomes in a murine setting has been demonstrated recently[4] to be effective in knocking down a central nervous system specific protein. These promising sets of data suggest that this technology is now emerging allowing targeted use of extrinsically generated EVs in order to counteract tumors.

Conclusion and outlook

Cumulatively, the studies briefly described make a resounding case for the involvement of EVs in all stages during cancer development. However, most of the aforementioned results are gathered from tissue culture experiments generating non physiological vesicle concentration levels. Therefore, it would be vital to substantiate these findings in more rigorous *in vivo* settings. These undertakings are currently hampered by considerable gaps in our knowledge of EV biogenesis and a lack of available *in vivo* tools[75]. It is interesting to note that, although EV formation occurs in all cells, most of our knowledge about their function stems from cells that have adapted to malignant transformation, while our knowledge about their roles in healthy tissue homeostasis lags behind. We have discussed the release and reception of cargo containing signaling molecules, as well as metabolic and

growth regulators, shuttled between tumor cells and their surrounding microenvironment. In this regard, it is the abundance or rather the delicate mixture of these molecules that charge EVs with cell transforming "superpowers". Like Trojan horses they may cross the cell barrier and reprogram cellular functions in favor of the malignant cells. However, these properties also make them formidable candidates for cancer diagnostics as well as for novel therapeutic approaches. Firstly, their composition may hold important clues about the type and stage of various types of cancers and also reveal possible new targets. Secondly, they could potentially be designed for the purpose of targeted intervention including the stimulation of local autoimmune responses or for the 'trapping' of disseminating cancer cells. Thirdly, during cancer treatment, EVs may switch their composition and may therefore exhibit traits for 'real-time' monitoring of therapeutic efficiency. However, while we make incremental progress in exploring all those possibilities many questions remain still unresolved. In particular those concerning their biogenesis, cargo selection and loading, as well as the mechanisms involved in their uptake, cargo liberation and incorporation into the context of the recipient cells. The incentives to investigate the functional connotations of EVs promise to change our understanding of cancer biology and potentially of how to tackle this complex set of diseases.

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Conflict of Interest

The authors declare no conflict of interest.

Figure legends

Figure 1 EV biogenesis. EVs can form from the endomembrane system or through budding/blebbing from the plasma membrane. The best-described pathway for the production of exosomes starts at the plasma membrane through endocytosis at cholesterol enriched lipid raft domains. The subsequently generated early endosomes (EE) fuse in a number of fusion events and concomitantly mature to late endosomes (LE) that can then form intraluminal vesicles (ILVs) by invaginations and pinching of the limiting membrane. The product is referred to as a multi-vesicular body (MVB). MVBs are then either destined for the fusion with the lysosomal compartment leading to cargo degradation or are tagged for fusion with the plasma membrane thereby releasing ILVs, thereafter called exosomes. The orchestrated redistribution of membrane lipids, sphingosine metabolites[10, 76] and/or the ESCRT machinery[33] have been reported to have crucial functions in exosomes and MV biogenesis. Large Oncosomes derived by membrane blebbing can be artificially induced through knock-down of the cytoskeletal protein DIAPH3[52].

Figure 2 Schematic representation of the flow of information regulated by EVs and LOs during metabolic reprogramming. EVs from glycolytic cancer cells can contain information that is fed to malignant or non-transformed cells (of cancer or stromal origin) and cause metabolic changes. For instance significant alterations can be induced in CAFs that in turn respond by the release of EVs containing sufficient material to sustain the cancer cell metabolism. This intercellular reprogramming evidences the dependency between the tumor and its adapted microenvironment whereby EVs can be seen as outsourced 'investments' undertaken to deliver metabolites and other material that promote tumor growth.

Figure 3 EV-mediated transfer versus the secretion of soluble molecules bound for ligand/receptor interactions. Local diffusion of proteins such as cytokines, chemokines or growth factors (exemplified for tumor to endothelial cells delivery) allows the engagement with their respective receptors on proximal located cells. In contrast, tumor cell-derived EVs allow the transfer of diffusible factors but also that of receptors, intracellular signalling mediators and RNAs all protected from degrading enzymes in the microenvironment allowing systemic transport via bodily-fluids such as blood or the lymph for their distribution. Thus, EVs can transfer their content not only to neighbouring stromal cells but also to potentially remote locations of future metastatic sites. The delivery of EV cargo to target cells may circumvent the necessity of specific ligand/receptor interactions.

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Figure 1

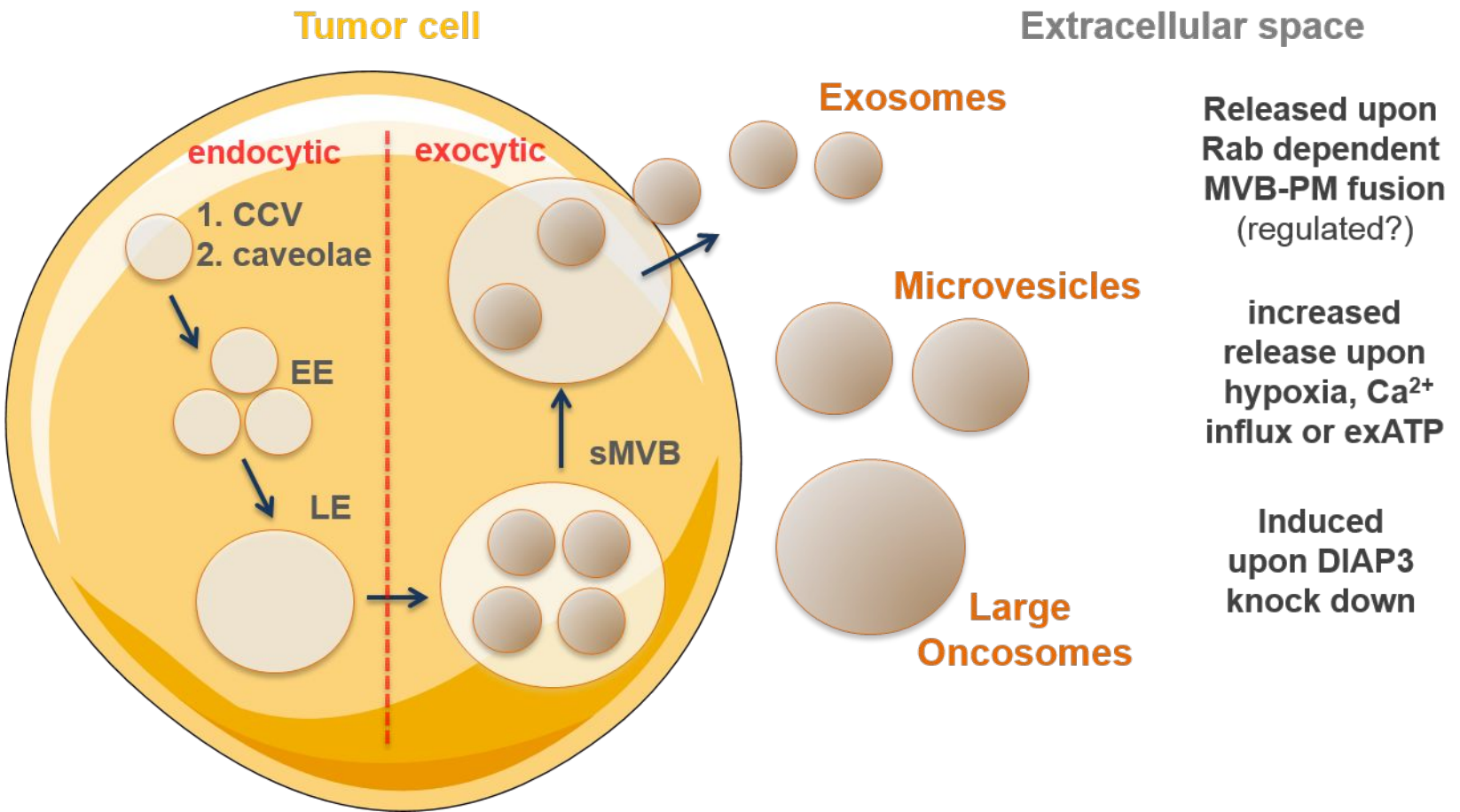

Tumor cell
Extracellular space
endocytic
exocytic
1. CCV
2. caveolae
EE
LE
sMVB
Exosomes
Microvesicles
Large Oncosomes
Released upon Rab dependent MVB-PM fusion (regulated?)
increased release upon hypoxia, Ca2+ influx or exATP
Induced upon DIAP3 knock down

Figure 2

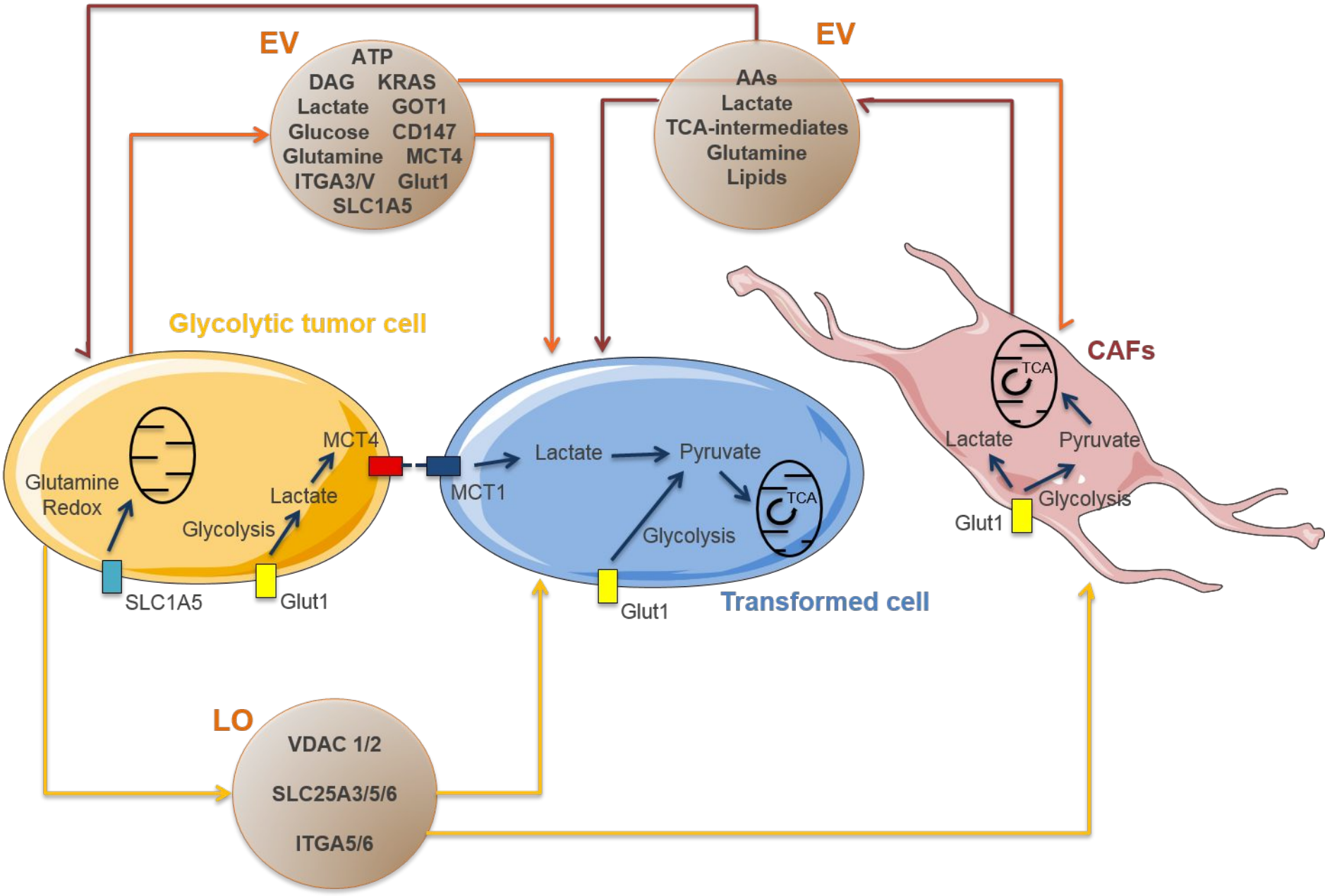
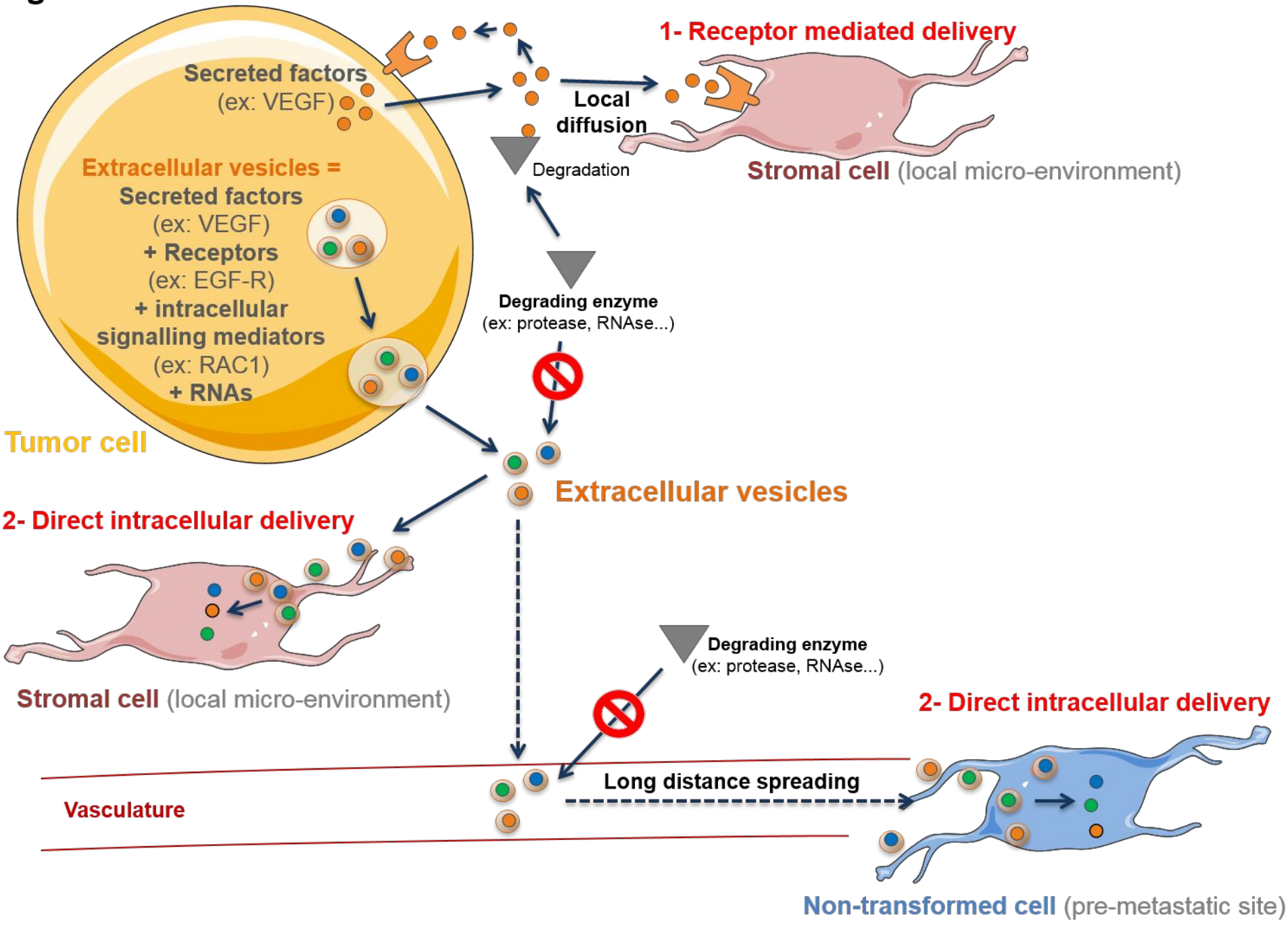

EV
ATP
DAG KRAS
Lactate GOT1
Glucose CD147
Glutamine MCT4
ITGA3/V Glut1
SLC1A5
EV
AAs
Lactate
TCA-intermediates
Glutamine
Lipids
Glycolytic tumor cell
Glutamine
Redox
MCT4
Lactate
Glycolysis
SLC1A5
Glut1
MCT1
Lactate
Pyruvate
TCA
Glycolysis
Glut1
Transformed cell
CAFs
TCA
Lactate
Pyruvate
Glycolysis
Glut1
LO
VDAC 1/2
SLC25A3/5/6
ITGA5/6

Figure 3


Secreted factors
(ex: VEGF)
Extracellular vesicles =
Secreted factors
(ex: VEGF)
+ Receptors
(ex: EGF-R)
+ intracellular
signalling mediators
(ex: RAC1)
+ RNAs
Tumor cell
1- Receptor mediated delivery
Local
diffusion
Degradation
Stromal cell (local micro-environment)
Degrading enzyme
(ex: protease, RNAse...)
Extracellular vesicles
2- Direct intracellular delivery
Stromal cell (local micro-environment)
Degrading enzyme
(ex: protease, RNAse...)
2- Direct intracellular delivery
Vasculature
Long distance spreading
Non-transformed cell (pre-metastatic site)